

The University of Chicago

The Changes in Amount and Distribution of the Iron-containing Proteins of Nerve Cells Following Injury to their Axones

A DISSERTATION

SUBMITTED TO THE FACULTY
OF THE OGDEN GRADUATE SCHOOL OF SCIENCE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY

BY

F. M. NICHOLSON

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THE CHANGES IN AMOUNT AND DISTRIBUTION OF THE IRON-CONTAINING PROTEINS OF NERVE CELLS FOLLOWING INJURY TO THEIR AXONES

F. M. NICHOLSON

The Hull Laboratory of Anatomy, The University of Chicago

FIVE PLATES (EIGHTY-NINE FIGURES)

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INTRODUCTION

During the past three decades an extensive literature has been written concerning the iron-reacting substances in cells of plants and animals of various species, wherein not only its presence, but also its location, both intracellular and extracellular, its assimilation, its physiological rôle, and its elimination from the cells have been considered. In general, iron-containing material

has been described in practically all cells thus far studied, including nerve cells. Although these studies have been descriptive in nature for the most part, in some instances the variations in amount and distribution of the iron-holding protein in different conditions have not been overlooked. A thorough study of these variations has not been made heretofore. It has even been questioned by some whether or not an iron-reacting material exists in cell constituents at all, though most investigators seem not to doubt its presence. The earlier researches upon cellular iron-containing proteins may be discredited to some extent because the methods and technique employed were not standardized to the extent where they were always reliable for obtaining uniform results, and again, the chemistry of the reaction, the method of distinguishing between organic and inorganic iron and the method for dissociating, that is, of unmasking the iron from organic materials, were not thoroughly understood.

The classic researches of Macallum ('91) upon the iron-holding substances in plant and animal cells stand out foremost as regards extensive, thorough and clean-cut experiments. For the first time the method of unmasking iron from, and demonstrating its presence in, organic materials, and the chemistry of the iron reaction were satisfactorily mastered. Since then the methods for investigation have not been improved except in minor details. Macallum's work was extended to include other tissues and cells, both plant and animal, but the findings have only been corroborated. To be sure, some work has been done recently upon the physiologic rôle of iron in chromatin, but these results need to be substantiated. As far as the theories concerning the function of iron are concerned, there seems to be little to offer for or against them.

In nerve cells, the phenomenon termed chromatolysis may be merely a physical change in the cytoplasm resulting in a change in staining reaction of the Nissl substance (Carlson, '02-'03) or there may be a chemical change involved. If it can be shown that the amount of iron-reacting protein demonstrated by a reliable microchemical method is changed by an experimental procedure, then we will have some evidence that a chemical change has taken place in the cytoplasm.

This work was begun at the suggestion of Dr. G. W. Bartelmez as an attempt to throw more light on the character of the axone reaction. Dr. Bensley pointed out the need for a microchemical investigation of the Nissl substance under these conditions. In such a study we are dealing with specific chemical substances, i.e., the iron-containing proteins, and the question as to whether the Nissl bodies are present as such in the living cell becomes a minor matter. The investigation of functional changes in the iron-holding material of nerve cells has been approached in this study through the examination of the changes which take place in the distribution of the iron of the iron-holding proteins of the nerve cell during and following injury to its axone.

It is with pleasure that I acknowledge my indebtedness to Prof. R. R. Bensley for his valuable direction in this work, to Prof. C. Judson Herrick for the aid received from him, to Prof. G. W. Bartelmez for his coöperation, and to Professors Preston Kyes, A. J. Carlson, and F. C. Koch for suggestions received from them.

1. Object and statement of problem

It is the purpose of this investigation to answer as fully as possible the following questions which have arisen from time to time: 1) Is the iron-containing material of the nerve cell identical with the basophilic or Nissl substance? 2) What is the behavior of the former after injury to the axone of the motor cell? 3) Does iron-containing material pass from the nucleus into the cytoplasm and from the cytoplasm into the axone hillock during the reaction to injury? 4) How soon after injury to the axone does the cell show changes in distribution of the iron-holding substance? 5) At what stage of the reaction are the maximum changes manifest? 6) Just when and in what manner does the cell begin to regenerate? 7) What is the range of variability during the degeneration and regeneration stages? 8) How long does it take for the cell to recover completely from injury? 9) And, finally, what are the differences in the various cellular reactions to axone injuries of a mild nature, such as ligation of the axone, as compared with the changes in more severe injuries, such as tearing out the nerve, which are sure to kill the nerve cells?

2. Axone reaction

When the axones of nerve cells are injured there are certain changes in the structural elements of the cell's cytoplasm which are commonly referred to as 'the axone reaction.' These changes have been studied in connection with a variety of methods of injury such as cutting, tearing, or compressing the nerve, poisoning it with toxins, stimulating it electrically or chemically, etc. They have also been studied in infections, inflammations and asphyxia of the nerves. In recent years fatiguing the cells has been a much used method. In all these conditions the axone reactions are similar. The differences are due to variability in degree of injury and time allowed for the reaction, as well as to complications associated with them.

In the past the study of axone reactions has been confined to the morphologic changes in the cytoplasm, nucleus, nucleolus, Nissl bodies and the canalicular apparatus. In the main the reactions to injury resulted in shrinkage of the cytoplasm, chromatolysis of the Nissl substance, distortion of the nucleus followed by excentricity of the same, change in volume of the nucleolus and fragmentation of the canalicular apparatus. The first and most complete changes have been described as occurring in the cytoplasm nearest the axone hillock where the Nissl bodies become dispersed and then disappear in a progressive manner from the nucleus toward the periphery and in a manner parallel with the degree of injury. When the cell does not degenerate completely as a result of the injury, the Nissl substance is eventually replaced around the nucleus by an accumulation of the material which is gradually reformed into normal Nissl bodies.

These morphologic changes show, in the last analysis, nothing other than that the staining reactions of the cells are changed. This may be interpreted to mean that the Nissl substance which is a nucleoprotein, possibly containing some carbohydrates and fats in the molecule, is necessary to the cell's metabolic equilibrium. When that equilibrium is disturbed by injury to the cell or its components, these complex molecules may become

depleted. Nothing is known about the chemical changes. It remains to be seen from this investigation that the study of the iron of these molecules lends evidence to a chemical change in the phenomenon.

REVIEW OF THE LITERATURE

Though the literature on the study of the iron-reacting proteins in plant and animal tissues is extensive, the greater portion of it is devoted to the study of these iron-containing substances in tissues such as blood and egg yolk, organs like the testes, thymus, pancreas and spleen, cells of the liver, carcinoma and bacteria, biochemical materials like casein, vitellinic acid and nucleins, rather than to the iron-holding materials in nerve cells. In addition to the above, the feeding of organic or inorganic iron-containing materials has been studied with the object in view of finding out where and in what tissues they were deposited. In this review, however, attention will be given only to the development of the methods used for the detection of iron and to the iron-holding proteins as these have been observed in nerve cells or nerve tissue.

As early as 1845, Vogel (Zaleski, '91) used the ammonium sulphide method in demonstrating iron in organs. Quincke ('96) says that in 1850 Mayer first used this method to demonstrate iron in the intestinal wall, and, that he himself at first used the ammonium sulphide and the potassium ferrocyanide plus HCl methods in his tests for iron in tissues. Perls ('67) repeated these methods and Bunge ('84) showed that ammonium sulphide unmasked organically bound iron, for which purpose he later employed a mixture of 90 cc. of 96 per cent alcohol and 10 cc. of 25 per cent HCl—better known as Bunge's fluid. A marked reaction for iron was then obtainable.

A new impetus in the study of iron was started when Zaleski ('86) used ammonium sulphide plus glycerine and potassium ferrocyanide plus HCl for the demonstration of inorganic iron in the liver. The ammonium sulphide and potassium ferrocyanide methods were also used by Macallum ('91) in studying organically bound iron in the ovary of *Erythronium americanum*. He showed that ammonium sulphide separated iron from the isolated chromatin, and he also employed sulphuric acid instead

of HCl in using Bunge's fluid. Finally he preferred potassium ferrocyanide instead of ammonium sulphide as the method of coloration of the iron. These results led Macallum ('91) to the conclusion that if iron is present in the intact nucleus it is so firmly bound in the molecules of chromatin that the ordinary reagents cannot unmask it, or it is so small in amount that its coloration is invisible under the microscope.

In the study of fresh tissues Macallum ('96) applied 2 per cent potassium ferrocyanide for an hour or more and then 5 per cent HCl for the detection of inorganic iron. To differentiate this from organic iron he incubated the tissues at 60°C. with aliquot parts of a solution of ammonium hydrogen sulphide and 50 per cent pure glycerine, after which the sections were placed on slides for a few days. The organic iron was then demonstrated as a greenish-black ferrous sulphide. Later Macallum used sulphuric acid in preference to HCl which does not so well insure the removal of inorganic iron.

Macallum ('97-'98) improved upon his old method by a new one in which the detection of organically bound iron requires freshly prepared 0.5 per cent aqueous hematoxylin solution which is pale yellow in color. The ferric ions, having been unmasked from the organic compound previously with sulphuric acid-alcohol at a temperature of 37°C. for from 1 to 36 hours or longer, unite with the unoxidized hematoxylin and form an iron-hematoxyline which precipitates as a blue-black compound. When the iron is unmasked in the tissues the ferric ions remain where they were unmasked and the color reaction is an indication of their exact location in the chromatin. It is easily demonstrable in very small quantities and Macallum and others claim this method to be by far the most accurate method for the demonstration of iron that is known.

It was of interest to find that Molisch ('92) had said that the potassium ferrocyanide method for the demonstration of iron is one of the finest reactions in chemistry.

In repeating Macallum's work in part, Gilson ('92) using HCl, sulphuric acid and sulphurous anhydride for unmasking the iron, claimed that these reagents were the best. He then applied the

potassium ferricyanide method for obtaining the Turnbull blue reaction and the potassium ferrocyanide method for the Prussian blue reaction.

Petit ('92) used 1 per cent HCl in absolute alcohol to free the organically bound iron in nuclein of the barley grain, after which he applied heat for 6 hours and obtained a good iron reaction upon coloration.

In 1893, Petit separated an iron-holding nuclein from barley maltcombs by extracting the pulverized material with 1 per cent potash solution at 60°C. for several minutes, filtering under pressure, neutralizing with dilute HCl, washing with water, alcohol and ether and then drying with sulphuric acid.

Hall ('96) used alcohol containing a little commercial ammonium hydrosulphide for unmasking the iron. This method was used later by Landau ('02) also.

In the potassium ferrocyanide plus HCl and the ammonium sulphide plus glycerine methods for the demonstration of iron, various fixatives such as alcohol, formol-alcohol, formol, formol-Müller, corrosive sublimate and Zenker's fluid may be used, according to Tracy ('05), but it is the consensus of opinion that alcohol gives results less open to criticism.

Brown ('11) employed a new method when he studied the iron content of hemoglobin, hematin and hemin. The colored compounds named were oxidized with hydrogen peroxide, thus forming colorless materials, after which he applied the potassium ferrocyanide plus HCl method. This method was applicable to dried blood, blood fixed with heat, alcohol or formalin, of which the latter fixative seemed to give better results. He also used the ammonium sulphide and glycerine method with good results. Paraffine or celloidin embedded specimens gave as good results as fresh tissues, although there was a tissue variation, because some tissues required a longer period of oxidation than others.

According to his text Abderhalden ('12) fixed tissues in 70 per cent alcohol for twenty-four hours, dehydrated with alcohol and then placed them in a mixture of ammonium sulphide and 50 per cent pure Molisch glycerine at 60°C. for from 1 to 14 days, after which they were embedded in paraffine. Sections

were placed for from 1 to 4 days in 4 parts of sulphuric acid and 100 parts absolute alcohol at 25°C. for the purpose of liberating the iron from the proteins. They were then washed in absolute alcohol and placed in equal parts of 0.5 per cent HCl and 1.5 per cent potassium ferrocyanide for half an hour, in which the Prussian blue reaction took place with the liberated iron.

Molisch ('13) showed that iron could be precipitated as a ferri-ferrocyanide (Berlin blue) by the addition of the basic salts from blood to a solution of ferrocyanide of potassium in the presence of 5 per cent HCl. He also used other methods such as with Rhodankalium, ammonium sulphide, etc. The same methods were used by Molisch for the demonstration of organically bound iron which had been unmasked previously with an acid alcohol.

After using all the methods for the microchemical detection of organically bound iron Moore ('14) found that the Macallum hematoxylin method surpasses all others in reliability and delicacy of reaction. When employing this method for organically bound iron one must be exceedingly careful that no inorganic iron is present. In order to use the method most effectively, it is necessary to avoid all traces of alkali and acid, since these interfere with the delicacy of the reaction.

Still later Mawas ('19) described a newer method for coloration of iron in tissues in which he used alizerin monosulphonate of sodium. He says that the ammonium sulphocyanide and the ammonium sulphide methods are inconstant in their reactions and cannot be relied upon on account of the solubility of the iron compound formed. The Prussian blue method is accurate and dependable because the iron compound obtained is insoluble. In describing the hematoxylin method he says that it is reliable and gives the most intense color reaction of all.

So far as I have been able to find Mackenzie ('97) was the first to report the presence of organically bound iron in nerve cells. Mackenzie said that the Nissl granulations in nerve cells are distinctly iron-holding, and consequently related to the iron-holding chromatin of the nucleus. In pathologic cells from rabbits which had been inoculated with rabies virus, it was found

that as long as basophilic granulations were present in the cell, iron-holding material was also present. In cortical motor cells from a rabid animal, it was found that oxyphilic granules appeared in the places where the Nissl granulations had been, but that these oxyphilic granules were but slightly iron-holding. The author says that it seemed probable that there was a conversion of the iron-holding basophilic granules into oxyphilic granules containing very little iron. In his report the following year Mackenzie (Macallum, '98) merely made reference to the presence of iron-reacting proteins in nerve cells.

Weber ('98-'99) added to the information already at hand when he found iron-containing substances in the ganglionic nerve cells from a boy who had died from meningitis.

In repeating Macallum's method on the nerve cells, Scott ('99) said that besides the Nissl substance, the covering of the nucleolus and the oxyphilic nuclear chromatin also contain iron. According to him the germinal cells of the nervous system in 7 mm. to 18 mm. pig embryos show that all the iron-holding material is confined to the chromatin of the nucleus, but that in later stages the basophilic iron-holding chromatin goes into the cytoplasm while the oxyphilic iron-containing material stays in the nucleus.

The fact that alkalis and acids affect the Nissl bodies so that no iron reaction is obtainable in them while the oxyphilic granules in the nucleus remain unharmed, as shown by Scott ('99), indicates that these structures—though they both contain iron—are chemically different. The iron-containing Nissl substances are soluble while the iron-containing oxyphilic nuclear materials are insoluble in alkali. In the embryo Scott found that the nuclei of nerve cells are much richer in the chromatin material which stains with basic dyes than after the Nissl bodies have become differentiated. He found, by use of the microchemical Prussian blue method, striking pictures indicating a transfer of iron-containing material from the nucleus into the cytoplasm during this stage of differentiation. Such a transfer has been assumed in various dividing cells on the basis of ordinary histological preparations. Scott remains the only one who has used a microchemical method on neuroblasts. The iron-containing nuclein compounds

are derived from pr  existing ones, which, in the mitosis of cells, become confined entirely to the nuclear chromatin.

It was observed by McCarthy ('00) that in the ganglion cells near small hemorrhages there was obtained a marked reaction for iron. It appears from this work that the iron must have been inorganic in nature, because none was unmasked from the cell cytoplasm. Later McCarthy ('07-'08) studied the nerve cells in the neighborhood of hemorrhages in the spinal cord and found that the cells absorbed the iron from the hemosiderin or else the hemosiderin itself, because he was able to show the iron of the hemosiderin in the cells and tissues.

Guizzetti ('15) demonstrated iron-containing materials in the cells of putamen, globus pallidus, substantia nigra and other parts of the central nervous system.

MATERIAL, METHODS AND TECHNIQUE

This study was not undertaken without realizing the obstacles that would have to be met. The animals used would have to be of a species conveniently housed, easily handled, easily kept in good health, easily operated upon and in which infection could be relatively easily avoided. For study of the changes in distribution of the iron-holding proteins in nerve cells after axone injury animals would have to be of a kind in which the span of life is relatively short, in which good nerve injury reaction could be obtained and in which the nerve tissue could be easily removed when the animal is sacrificed. In order to prevent artefact changes which are produced so easily in nerve cells, the technique had to be developed in operation upon the animal, procuring the tissue after death, proper fixation, dehydration, clearing, embedding, sectioning and applying the methods for the demonstration of the iron, etc. The technique in all these stages was tried repeatedly in the employment of various methods and many modifications of them.

Common white rats, *Mus norvegicus albinus*, were used in these experiments. These were purchased from breeders, from whom a history of the animals was obtained. They were young adults, of either sex, normal and weighing about 175 grams each. During

the time of the experiment they were kept in a suitable animal room and fed with appropriate foods to maintain weight and prevent deficiency diseases. From day to day they were handled so that they could be brought to the operation without being frightened.

When ready for operation the rat was etherized to complete relaxation, after which it was prepared for operation. This was carried out aseptically in every stage and, out of more than 200 such operations, infection was encountered but twice.

After the hypoglossal nerve had been exposed carefully, it was freed from its surrounding connective tissue with precaution so as not to cause traction on the axones. When freed in this manner a ligature was carefully placed around the nerve and the tying was done equally carefully. In the case of tearing the axones the nerve was freed in the manner described above, after which it was torn from its deeper moorings. In each animal studied the nerve was ligated at a point midway between the point of exit of the nerve from the skull and the attachment of the posterior belly of the digastric muscle to the hyoid bone.

Within a week the wound was healed and in from 10 to 14 days the stitches were absorbed. The animal was kept under normal conditions until the desired time for nerve cell reaction had expired.

When the time allowed for axone reaction had passed the animal was killed by etherization, followed by free bleeding from the carotids and jugulars to drain the blood from the tissues so that finer dissection could be made.

The nucleus of the hypoglossal nerve was selected for study because of the relatively easy access to the nerve for operation, because it is almost a pure motor nerve so that sensory phenomena could be eliminated, because operations upon this nerve result in no ill effects to the animal and because we know just what structures the nerve supplies. For the latter reason the atrophic changes in the organs supplied can also be studied. In the rat the hypoglossal nerve lies parallel with, and lateral and deep to, the posterior belly of the digastric muscle. Other reasons for selecting this nerve are that, when the animal has

been killed, the medulla can be readily and quickly removed without injury to the nuclei, that the medullary tissue is one that is easily penetrable by fixatives and that the hypoglossal nerve can again be dissected out and prepared for the study of changes in it. Lastly, but for reasons perhaps more important than the foregoing, the medulla can be easily oriented in the embedding process so that the cells can be sectioned parallel with the stream of axones, thereby cutting through the axone hillock in many of the cells. The hypoglossal nuclei can be located without difficulty, the normal and experimental sides can be fixed alike, sectioned with the same sweep of the knife, stained alike and studied side by side in the same preparation.

1. Specific technique used for demonstration of iron-containing substance

Care was exercised to be certain that the glassware, alcohols, sulphuric acid, etc., were free from inorganic iron.

In the study of the distribution of the iron-reacting substances in the nerve cells the hematoxylin method devised by Macallum ('97-'98) was employed. Results with it were verified, however, with those obtained with the Prussian blue method.

Although fixation in 70 per cent alcohol was employed by Macallum, it was found that fixation in 95 per cent alcohol, suggested by Bensley, was just as good, but by varying the time of fixation it was found that better results were obtainable after forty-eight hours' fixation than by a shorter time. The medulla of the rat being about 6 mm. in width at the level of the nucleus hypoglossi, it became necessary to procure the piece of medulla containing the nucleus, so as not to be too thick for fixation. This was readily accomplished when it was found that the greatest extent of the two nuclei was less than 2 mm. Therefore, a piece of the medulla containing the nuclei, but about 2 mm. in thickness, was cut out.

Thorough dehydration was effected with absolute alcohol in from 2 to 5 hours, after which the tissue was cleared in cedarwood oil until transparent. After two changes in paraffine and em-

bedding, sections were cut not more than $7\ \mu$ in thickness. They were then mounted on slides, deparaffinized and passed through alcohols to a solution of 4 volumes of pure sulphuric acid and 96 volumes of 95 per cent alcohol kept at 60°C . for from 5 to 60 minutes. Better results were obtained at this temperature for the shorter time than at 37°C . for the longer period as Macallum did. After the iron had been liberated from the proteins by the acid alcohol the specimens were washed in 95 per cent alcohol and passed through graded alcohols to water, preparatory to the microchemical reaction. For this, freshly prepared aqueous hematoxylin was applied for from 1 to 5 minutes in which time the blue-black iron-hematoxylin reaction takes place. The hematoxylin must be of excellent quality, it must be freshly prepared and unoxidized and the solution must be pale yellow in color. Such a solution does not stain chromatin which has not been treated with the acid alcohol. It is true that this solution will give a similar reaction with inorganic iron, but such iron is not present in nerve cells when the proper precautions have been carried out. Counterstaining with a dilute alcoholic solution of erythrosin was made as a contrast against the blue-black iron granules. Dehydrating, clearing and mounting in neutral cedarwood oil was then done to procure permanent specimens.

In each ligation experiment the normal side of the nucleus hypoglossi was compared with the injured side as regards distribution of the iron-containing proteins, and typical cells of both sides drawn. Likewise Nissl preparations were compared with iron preparations, and control specimens in which the iron had not been unmasked gave negative results.

The sections selected for most careful study were taken approximately midway between the upper and lower (spinal) ends of the hypoglossal nucleus. Except for an occasional cell toward the dorsal or medial side of the nucleus (which were unaffected), all the cells in any one operated specimen were in practically the same phase of degeneration or regeneration, as the case might be. From these cells those were selected for drawing in which the plane of section passes through both the axon hillock

and the nucleolus. Many more cells were drawn than those presented in the plates, and of these those were selected for publication which are most typical of the designated days of reaction. As the figures show, there is some variation from a uniform sequence of changes from day to day, probably due to differences in age, size and susceptibility of the rats employed (in spite of careful control of these matters) or to minute differences in distance of point of ligation of the nerve from the hypoglossal nucleus. But these deviations from a uniformly progressive series of changes are relatively slight. Degenerative changes progress with great regularity during the first fifteen days of reaction to axon injury, after which further degeneration (in cases where axons were torn out) or regenerative changes (in the case of ligated axons) follow also in regular sequence.

2. The iron reaction

The microchemical reaction for iron in the protein molecule of chromatin is one in which there is an unmasking of the iron from the proteins by acid alcohol and a chemical reaction between the ammonium sulphide, potassium ferrocyanide or hematoxylin (as the case may be) and the iron which produces a colored compound visible under the microscope. With ammonium sulphide the color is black, with potassium ferrocyanide it is blue, and with hematoxylin it is blue-black.

These methods have been improved upon from time to time until, at present, it seems to be the consensus of opinion that iron can be demonstrated unquestionably by experienced technicians—whether the iron is organic or inorganic. Inorganic iron may be demonstrated without any preliminary treatment, but in order to demonstrate organically bound iron the chromatin material must first be subjected to acid-alcohol as described, in order to unmask the iron from the nuclein or nucleinic acid. When it is so unmasked it is liberated in a form not unlike inorganic iron. In other words, it is ionized.

In such microchemical methods the color reaction is due to a purely chemical reaction between a chemical on the one hand

and the tissue element on the other. A prerequisite for such a reaction is that the tissue element must be so fixed that its chemical properties are preserved. Examples of such reactions are the Prussian blue reaction for iron, the Millon reaction for proteins containing the monohydroxy-benzene nucleus, the iodine reaction for starch and the blue-black reaction between iron and unoxidized hematoxylin. Microchemical reactions indicate the exact quantity and location of the material sought and in the exact place where it is in the freshly fixed cell plasma.

In contrast to the microchemical coloring methods there are the physical staining methods in which the process of coloration is probably one of adsorption of the dye by the cellular structure in question. In physical staining the results vary, probably depending upon differences in colloidal concentration, size of colloid particles, and aggregates of particles, whether or not the sol or gel phase is present, and differences in surface tension of the structure being stained, as well as upon differences in size of the dye particles (cf. Bayliss, '15, pp. 70, 71). No doubt these methods are variable again, depending upon whether or not the nature of the cell structures has been altered by acidity or alkalinity resulting from postmortem changes or from the fixatives employed. Physical staining is accomplished whether the dye used is acid or basic. While physical methods are excellent for differential staining (and in most cases they are the best at hand) there is an outstanding objection to them, and that is, that one never knows exactly the true physical nature of the particular structure under observation. This may not be seen at all, or it may be small, or it may be large, depending upon the technique. For example, different descriptions have been given of the Nissl bodies. Probably many of these differences were due to variations in technique under which the physical structure was alterable. Furthermore, it has been questioned whether such structures as Nissl bodies exist at all as such, because they are not seen in the living protoplasm, but rather after they have been coagulated. The Nissl method of demonstrating them is a physical one.

3. Additional proof of nerve injury

During the progress of the study of the changes in the iron-containing materials in the nerve cells it became desirable to know for a certainty whether or not the protoplasmic continuity of the axis cylinders was interrupted. In every animal macroscopic study revealed food that had been collected under and at the side of the tongue on the side of the ligated nerve. It was also noted that this half of the tongue was anemic and flaccid as compared with the normal half. Upon microscopic study it was observed that the muscle fibers of the injured side were atrophied considerably, depending upon the time allowed for reaction after the ligation. The atrophied side was also considerably smaller in cross section (fig. 1). A similar condition of the tongue was noted in instances where the nerve had been torn.

To complete the demonstration of the complete interruption of the nerve fibers, the nerve was dissected out after the animal had been killed and a piece of it, including a portion distal to the ligature and a portion central to it, were prepared for microscopic examination. Macroscopically it was noted that the distal part was not white in appearance like the central part. It was frequently seen to be larger than normal and edematous in character. Upon sectioning the nerve after silver nitrate impregnation the axis cylinders in the central portion were normal (fig. 2), while in the distal portion they were not discernible or else there remained a few granular axis cylinders (fig. 3), depending upon the time allowed for degeneration of the nerve.

Neuromas were seen in some of the preparations to have grown near the point of ligation (fig. 4).

Electrical stimulation of the ligated nerve showed that the distal portion had degenerated completely, as there was no response in the tissue normally supplied.

OBSERVATIONS

1. Distribution of iron-holding material in the normal nerve cell

The uninjured motor nerve cell from the hypoglossal nucleus after the iron procedure shows the cytoplasm, nucleus, nucleolus, axone and dendrites with their normal relationship to each other and with no shrinkage spaces surrounding the cell body (figs. 5 to 23). Irregularly shaped masses showing the iron reaction about the size and form of Nissl bodies were demonstrated in the cytoplasm. These bodies are sharply defined and are located in the cell in all its parts except in the axone hillock, but they are slightly larger and more numerous in the midzone between the nuclear membrane and the cell wall than elsewhere (fig. 19). The general cytoplasm with its contained iron-holding masses is sharply defined from the axone hillock in these preparations (figs. 8, 11 and 18).

The nucleus is always a large round body centrally located (figs. 14 and 19). It has no indentations into nor protrusions from its surface ordinarily. A small amount of material showing a slight reaction for iron is present in the nuclear membrane, especially on the side nearest the axone, and within the nucleus, where, in addition to the nucleolus, there are present masses of iron-holding substance which are variable in size and shape as well as in number. These are not stainable with toluidin blue, methylene blue nor other basic dyes. Usually these granules are smaller than those seen in the cytoplasm. They tend to be round (figs. 6, 12 and 19) and vary in number, as will be seen upon comparing figure 8 with figure 22. Though they are, as a rule, evenly distributed in the nucleoplasm (fig. 23) they may be seen grouped on the side nearest the axone (fig. 8). In some of the nuclei a large homogeneous mass of iron-reacting material may also be seen on the side closest the axone (figs. 5, 14, 20 and 23).

An iron reaction is obtained in the nucleolus—a round and sharply defined body which is either centrally located (figs. 6 to 9, 11 to 13, 16 and 17) or excentric in position (figs. 5, 10, 14, 15 and 18 to 23). In the latter case it is practically always seen in the side of the nucleus nearest the axone hillock. The

nucleolus is usually uniform in size providing it is cut through its center. A study of serial sections shows this to be the case. Tangential sections of it are naturally smaller. It always gives a distinct reaction for iron more readily and sooner than any other structure in the nerve cell. Early investigators employing the ammonium sulphide plus glycerine and the potassium ferrocyanide plus HCl methods also found this to be the case. The iron-containing substance in the nucleolus seems to be more concentrated and more easily dissociated than elsewhere in the cell.

The axone and its hillock are free from iron-reacting proteins so far as has thus far been determined. The axone hillocks vary in size from those rather small (fig. 9) to those much larger (fig. 18).

Like the Nissl bodies, the iron-holding masses are also present in the dendrites where they are longer, more slender, and not as large nor as numerous as those in the cell plasma (fig. 8). They are arranged parallel with the long axis of the dendrite.

The microscopic picture of the iron-containing masses corresponds precisely with that of the Nissl substance.

2. The relation of the iron-containing material to the Nissl substance

Under high magnification it can be seen that the Nissl bodies are not solid, but that they are composed of many smaller granules which are variable in size. Both the iron-containing material and the Nissl substance are basophilic in nature and are readily stainable by practically all basic dyes. They are similar to the nuclear chromatin and, according to Scott, derived from them. Alkalies dissolve out the Nissl material and the iron-holding substance is taken out with it so that neither is demonstrable after such treatment. The oxyphilic material in the nucleus is an iron-holding one, but it is not dissolved by alkalies.

In order to show the relation existing between the Nissl material and the iron-reacting masses demonstrated, camera lucida drawings were made of the iron-containing masses and Nissl substance in one and the same cell. A series of preparations was

stained with toluidin blue and erythrosin to demonstrate the Nissl bodies. Drawings of these were then made with the aid of the camera lucida. These same preparations were then destained by alcohol, the iron was unmasked by sulphuric acid and then demonstrated by the Macallum hematoxylin method. The same cells were again drawn by the aid of the camera lucida apparatus. A tissue paper copy of the one was superimposed upon the other. With the exception of a few small bodies the two sets of preparations agree in detail. We may therefore conclude that the Nissl substance is or contains an iron-holding protein. From the studies of Held, Macallum and Scott it is probable that the Nissl substance is a nucleoprotein. These drawings are shown side by side in figures 84 to 89. Figures 84 and 85 represent the normal cell, figures 86 and 87 represent a cell whose axone was ligated, and figures 88 and 89 represent a cell whose axone was torn. It need hardly be said that the figures here presented showing iron-holding protein correspond strikingly with the typical Nissl picture. They show at a glance that the iron-reacting material is in the Nissl bodies and besides it is in the nucleolus and in the oxyphilic chromatin material of the nucleus. In the case of a few small Nissl granules (figs. 86 and 87) near the periphery of the cytoplasm and between the larger granules in the center of the cell there is no iron reaction. Perhaps in the latter cases the iron was more readily unmasked and diffused before the preparation was removed from the acid-alcohol.

3. Changes in distribution of the iron-reacting substance in the cell following ligation of the nerve

The changes in the distribution of the iron-containing material of nerve cells following ligation of their axones were studied after intervals of one day up to fifty days and thereafter at intervals of one month up to six months. In general, the degenerative changes which took place progressed gradually from the first day up to the fifteenth day (figs. 24 to 38) after which regenerative changes took place and progressed gradually from the sixteenth

day up to the forty-fourth day after ligation (figs. 38 to 65). The degenerative changes are characterized by gradual decrease in iron-holding proteins in the cytoplasm and nucleus, distortion of the nucleus, and, after fixation, shrinkage of the cytoplasm. The regenerative changes are characterized by the gradual increase in iron-reacting material in the cytoplasm and nucleus and return of the nucleus and cytoplasm to normal. These two phases may be divided into substages which occur as follows: First to third day, fourth to eighth day, ninth to fifteenth day, and sixteenth to forty-fourth day.

a. The cytoplasm. Twenty-four hours after ligation of the axones the iron-containing masses in the cytoplasm of the cells begin to disappear all around the nucleus and close to it (fig. 24). This depletion progresses gradually from the nucleus toward the periphery up to the fifteenth day when it becomes most marked (figs. 25 to 38). In this degenerative phase the stage in which the cells are most affected is from the ninth to fifteenth days, as is shown in figures 32, 33, 35 and 38.

From the first to the fourth day there is, in addition to the disappearance of the iron-containing masses immediately around the nucleus ('central chromatolysis'), a decrease in their number in the rest of the cytoplasm and the appearance of masses of iron-containing material which are considerably larger than normal in the periphery of the cell (figs. 25 and 26).

From the fourth to the eighth day the disappearance of iron-reacting material from the cytoplasm is apparently halted and the large masses of iron-holding substance in the periphery of the cell disappear only to be replaced by numerous smaller ones (figs. 27 to 31) which exhibit an appearance of fragmentation (figs. 30 and 31). In this stage it is evident that more iron-containing substance accumulates in the neighborhood of the axone hillock (figs. 28, 29 and 31), though these masses may be as large as in figure 29 or small as in figure 31. This period from the fourth to the eighth day corresponds to the one following the first frantic efforts of the axones to regenerate as shown by Cajal ('08).

The most marked stage of all, which occurs between the ninth and fifteenth days as represented in figures 32 to 38, shows that

the depletion of iron-containing material from the cytoplasm continues peripherally at a very rapid rate (figs. 32, 33, 35 and 38). Instead of the numerous, scattered, small bodies of iron-holding substance in the periphery of the cytoplasm as shown in the preceding stage, there is here an accumulation of the iron-containing material close to the cell wall in the form of a more homogeneous mass with a few scattered granules (figs. 32, 35 and 38). Occasionally a cell is seen in which a large part of the periphery of the cytoplasm is free from iron-reacting protein (figs. 33 and 35). In this stage the accumulation of iron-holding substance about the axone hillock is more marked than in the preceding (figs. 32, 35 and 38) and the hillock itself appears to have become much smaller. Whether this decrease in size is real or merely due to shrinkage of the periphery of the cytoplasm, especially that about the axone hillock, is a question.

As the degenerative changes progress in the preceding stages the cytoplasm is not always left entirely free of granules giving the iron reaction. for occasionally large or small granules may be seen near the nuclear membrane (figs. 30, 34 and 37).

The regenerative changes begin with the sixteenth and continue to the forty-fourth day when the cell has become practically normal again so far as the masses of iron-containing material are concerned (figs. 39 to 65). Granules of iron-holding substance begin to reappear in the cytoplasm near the nucleus (fig. 39) and increase in number and size from day to day and, as will be seen by referring to the figures, the granules are at first small (figs. 39, 40 and 41), but become larger until they fill the rest of the cytoplasm gradually from the nucleus toward the periphery. While an increased amount is present around the axone hillock in the first few days of regeneration (figs. 40, 41, 47 and 49) this condition becomes typical in the later days (figs. 52, 54, 56, 59 and 69).

From the forty-fourth day up to six months after the axone injury the iron-reacting material in the nerve cells remains unaltered (figs. 65 to 76).

Besides the changes in distribution of the iron-containing protein another outstanding feature is the shrinkage of the

cytoplasm as displayed in fixed material, and probably due to increased water content. This begins some time during the first day of reaction (fig. 24) and becomes most marked from the ninth day (fig. 32) to the forty-fourth day (fig. 65), after which the condition again approaches the normal (figs. 65 to 76). In this connection the changes in the internal reticular apparatus of Golgi as described by Marcora ('08) are of interest.

b. The nucleus. From the first to the end of the third days of reaction to axone ligation there is a general depletion of iron-holding substance in the nucleus as is evident in the nuclear wall and in the decrease in number of granules in the nucleoplasm (figs. 25 and 26). In figure 25 there are two places where no demonstrable iron is present in the nuclear membrane. It will also be noted that the few granules present within the nucleus are located near the periphery rather than close to the nucleolus.

This stage is followed by one lasting from the fourth to the eighth days in which the nuclear iron-reacting material is again increased to normal. Although this is not evident in the nuclear membrane, it is marked by the greater number of iron-reacting granules uniformly scattered throughout the nucleoplasm (figs. 27 to 30) and the large diffuse masses in the side of the nucleus nearest the axone hillock (figs. 27 to 30), especially in figure 29. In figures 27 and 28 it will also be seen that the iron-containing protein is absent from the nuclear membrane at the pole opposite the axone hillock.

In the stage of most marked degenerative changes, namely, that extending from the ninth to the fifteenth days, the nuclear iron-reacting content again becomes markedly decreased (figs. 32 to 38) with such exceptions as are present in figure 33, where a large mass of iron-holding substance is collected in the nucleus near the axone hillock, and in figures 34, 36 and 37 where the amount of iron-containing material is practically normal. In figures 32, 35 and 38 the nuclear granules which give a reaction for iron are small in size, but scattered uniformly in the nucleoplasm. In figure 32 a few large granules are located near the nucleolus and in figure 38 they are present in the region of the nuclear membrane. In these cells there is no appreciable amount

of iron-reacting substance in the nucleus near the axone hillock and the iron-holding protein of the nuclear membrane has become almost entirely depleted. In practically all these cells (figs. 32, 33 and 35 to 38) the iron-containing material is absent from the nuclear wall in the pole farthest from the axone hillock.

Following this climax of axone-reaction changes in the distribution of the iron-reacting masses in the nucleus is a stage lasting from the sixteenth to the forty-fourth days, in which the iron-holding substance and its distribution again become normal (figs. 39 to 65). This is characterized by the reappearance of granules of various size from small to large and of various morphology from round granules to irregular masses which are uniformly distributed through the nucleoplasm (figs. 39 to 65). In figure 50 there is a predominance of small round granules, while in figure 52 the majority are large and irregular. The large mass of iron-holding substance in the pole of the nucleus nearest the axone also becomes a feature distinguishing the normal nucleus from that markedly affected (figs. 39, 41, 45, 48, 50, 52 to 57, 59, 63, 64 and 65). Likewise the iron-reacting material of the nuclear wall becomes normal (figs. 39 to 65), although there are a few cells in the early stages of the period of regeneration in which the nuclear wall of the pole farthest from the axone is still deficient in iron-containing protein (figs. 41, 43, 44, 46 and 48). Within this stage of regeneration there is present an interesting additional phenomenon within the nucleus (fig. 49) in which it appears that there is a distribution of iron-containing material from the nucleolus toward the periphery of the nucleoplasm, or a formation of this material around the nucleolus.

From the forty-fourth day up to the end of six months the nuclear iron-reacting substance remains normal as regards quantity, size and morphology of the granules, and distribution (figs. 66 to 76).

One important feature of the nucleus not described heretofore is its change in shape. During the greater part of the periods of degeneration and regeneration the nucleus becomes distorted and irregular in outline, as will be seen upon reference to figures 28 to 30, 34, 41, 42, 44, 46, 48, 51, 53, 56 to 58 and 60.

c. *The nucleolus.* The nucleolus itself remains as a round, definitely outlined body which is of uniform size when the same precautions are taken as outlined in the study of the nucleolus in the normal cell, i.e., when only median sections of it are studied. Although the nucleolus is approximately centrally located in many of the cells (figs. 27, 28, 31, 32, 37, 38, 40, 43, 44, 45, 48, 51, 55, 56, 58, 68, 69 and 71 to 75) throughout the stages of degeneration and regeneration, in many others it is decidedly excentric as in figures 26, 29, 30, 33 to 36, 39, 41, 42, 43, 49, 50, 52, 54, 57, 59, 64, 65, 67, 70 and 76. In most cells this nucleolar excentricity is toward the pole nearest the axone hillock, as in the normal cells, but there are a few exceptions to this (figs. 30, 33, 35 and 42). It will be noted that these exceptions occur in the stage of greatest cellular reaction. The position of the nucleolus probably has no significance whatever. It tends to settle with reference to gravity. This might occur while the brain was being exposed. The lowest part of each cell is roughly the axone pole. It was proven to settle with reference to gravity in the electric lobe cells of the Torpedo (Dahlgren, '15).

d. *The axone.* At no stage of the periods of degeneration and regeneration during the reaction of the cell to axone injury was iron-holding protein observed in the axone or its hillock. The hillock became much smaller in size, progressively, from the eighth day up to the fifteenth day of reaction (figs. 31 to 38), after which it returned to normal size gradually from the sixteenth to the forty-fourth days (figs. 38 to 65). In this period the most marked change in the size of the hillock is between the eighth and twentieth days (figs. 31 to 43). Associated with this decrease in size is a marked accumulation of iron-containing material in the cytoplasm immediately around the hillock (figs. 31 to 43). If the axone hillock became smaller, relatively, than the whole cell, then iron-containing material invaded the cytoplasm which normally is iron-free, i.e., part of the hillock itself. But since there is a shrinkage of the cytoplasm as a whole it is difficult to say whether or not there is an actual decrease in size of the hillock (figs. 24 to 62). From forty-four days up to six months the axones remained normal.

e. The dendrites. No pathologic changes were observed in the bodies of iron-reacting material in the dendrites nor in the dendrites themselves.

4. *The changes in distribution of the iron-reacting substance in the nerve cells following tearing of the axones*

It has long been known that nerve cells react differently to different degrees of injury. For instance, to mild forms of injury to the axones the cells show slight degenerative changes; to more marked injuries profound changes take place; and to injuries of still greater degree the cells degenerate completely and die. It was for these reasons that it was deemed advisable to produce an injury to the axones of such a nature that the cells would show extreme degenerative changes and to observe the changes in the iron-holding protein under such conditions as well as to compare them with changes observed in the ligation experiments. In this case the hypoglossal nerve was dissected from its surroundings just as in the operation for ligation of the nerve, but instead of ligating the nerve, it was torn or cut off as close as possible to the foramen. Striking differences were observed in the cells of these preparations as contrasted with those in which the ligation had been made. In view of these findings a series of experiments was made in which the changes were studied after five, ten, fifteen, twenty days and so on up to fifty days. In the cytoplasm the changes observed were a gradual reduction in the amount of iron-holding substance and shrinkage of the cell from the first to the twentieth days after injury. In the nucleus the reduction of iron-containing material occurred from the twentieth to the thirty-fifth days, when the cell was almost completely degenerated and the nucleus became distorted. Regenerative changes never took place in these cells.

a. The cytoplasm. In these experiments of tearing the axones the iron-reacting material in the cytoplasm diminishes gradually from the first to the twentieth days of reaction, when it has disappeared entirely (figs. 77 to 80).

After five days of reaction the iron-reacting granules are fewer in number and smaller in size than those in the normal cell although their distribution is normal (fig. 77), but the degree of reaction is not as great as it is five days after ligation of the axones (compare fig. 77 with 28). This is probably due to the absence of the great and usually transient regeneration activity in the first two to four days after injury (cf. Cajal, '08). At the tenth day the changes practically equal those ten days after ligation. Compare figures 78 and 33, where the cytoplasm is devoid of iron-holding protein except for a few granules in the region of the axone hillock. On the fifteenth day there is, likewise, almost complete loss of iron-containing substance from the cytoplasm (fig. 79). This is a much greater change than is evident at the fifteenth day of ligation (fig. 38). On the twentieth, twenty-fifth, thirtieth and thirty-fifth days (figs. 80 to 83, respectively) the iron-containing granules have disappeared. Forty, forty-five and fifty days after tearing the axone the cells had degenerated beyond recognition except for a few whose axones had not been included in the tearing and therefore remained unaffected. At no time is there an accumulation of iron-reacting material in the periphery of the cytoplasm nor about the axone hillock (figs. 77 to 83) as was observed in the ligation experiments.

Shrinkage of the cytoplasm was observed after dehydration just as in the ligation procedure except that it was not as marked in degree (compare figs. 77 to 83 with figs. 24 to 65).

b. The nucleus. It appears that the changes in the nuclear iron-holding protein are retarded somewhat as compared with those following ligation, although the iron-containing substance does disappear gradually from the twentieth up to the thirty-fifth days after tearing the axones.

After five, ten and fifteen days of reaction the nuclear iron-reacting substance remains normal in amount, distribution and size of granules (figs. 77 to 79). It is only after the twentieth day that changes occur in the form of an increase in amount and size of granules in the periphery of the nucleoplasm (fig. 80). This rapidly disappears by the twenty-fifth day when only a few

small granules remain (fig. 81). By the thirtieth and thirty-fifth days even these disappear (figs. 82 and 83) and thereafter the cells become so degenerated that iron-holding material is no longer demonstrable.

Disappearance of iron-containing protein from the nuclear membrane did not take place until after the thirtieth day (fig. 82) and grouping of iron-reacting material in the nucleus on the side nearest the axone was not observed either (figs. 77 to 83). Distortion of the nucleus as a whole, likewise, did not occur until after the thirtieth day of axone reaction (fig. 82).

c. The nucleolus. No changes from the normal were observed in the iron-holding material of the nucleolus. Up to the thirty-fifth day the size, shape and position of this structure were the same as in the normal or in the experiments where ligation was performed (figs. 77 to 83).

d. The axone. Under these conditions of tearing the axones, iron-containing substance was not observed in the axone nor in its hillock. The disappearance of iron-reacting protein from the cytoplasm was so complete that, except for a few granules seen in figures 77 and 78, no iron-holding material was grouped about the hillock (figs. 77 to 83), in fact it could not even be outlined. No change was observed in the form of the axone up to the thirty-fifth day.

e. The dendrites. The only change observed in the dendrites was the gradual but complete disappearance of iron-containing material from the fifth to the thirty-fifth days of reaction to the injury (figs. 77 to 83). After this period the cells were completely degenerated.

5. The similarity in the morphologic changes in the nerve cells in studies of Nissl and iron preparations

The changes in the size, shape and distribution of the iron-reacting granules in the cytoplasm and nucleus of nerve cells, together with the shrinkage of the cytoplasm and the distortion of the nucleus following ligation or tearing of their axones correspond identically with the morphologic changes observed in

studies of the Nissl substance in nerve cells following other types of injury as recorded in the literature and enumerated in this paper under the heading "The Axone Reaction" (p. 40). The Nissl bodies like the iron granules undergo similar changes. They become smaller in size and disappear progressively, beginning near the nucleus at the side nearest the axone and continuing peripherally while at the same time changing considerably in size and shape. They collect about the axone hillock in the most marked stages and reappear in the cytoplasm immediately around the nucleus and spread peripherally as regeneration begins. No doubt there are also changes in the nucleus, but since the oxyphilic nuclear chromatin, as distinguished from the nucleolus, does not stain by the Nissl method, such changes have not been described.

Cytoplasmic shrinkage and distortion of the nucleus take place at approximately the same time and to the same extent under both procedures.

DISCUSSION

There seems to be no question regarding the reliability of the microchemical tests for iron in plant and animal tissues. They have been employed unquestioningly by chemists and biologists since the first description of iron in cells by Vogel in 1845. Of these the chief methods used have been the ammonium sulphide plus glycerine, the potassium ferrocyanide plus HCl and the hematoxylin methods. Variations in technique have been centered mainly around that of unmasking the iron from the protein molecules, but as regards the iron reaction itself the technique has been similar throughout. When the methods were first used only inorganic iron was discernible. This was true in the study of iron in many kinds of cells, but in case of the nerve cells, where there is no free iron normally, the iron described has first to be dissociated from the molecule to which it is organically bound. In addition to the above-named facts the methods for microchemical detection of iron were checked, the one against the other, and it was found that the results coincided uniformly. The main advantage of one method over another is that the blue-

black reaction with the hematoxylin method can be seen better under the microscope than can the Prussian blue in the potassium ferrocyanide plus HCl method or the black in the ammonium sulphide plus glycerine method.

That an iron-containing substance is present in the cytologic structure of plant and animal cells there seems to be no doubt except that expressed by Mathews ('16, p. 176) who writes: "It appears, from some recent work, that iron, contrary to an earlier view, is not present in all nuclei" and that found in instances where the solutions or the glassware used may have contained inorganic iron.

Undoubtedly the nerve cells contain iron-holding protein in considerable quantity as compared with many other animal cells. Since it is the chromatin or chromidial substance in which the iron is found, this is to be expected when we consider the large amount of basophilic material (Nissl substance) in the cytoplasm, the chromatin or oxychromatin in the nucleus and nucleolus. This latter structure gives a reaction for iron more readily and more markedly than any other protoplasmic structure when subjected to the iron tests. Probably this iron-containing protein makes up an intricate and necessary part of cells so highly specialized as nerve cells. Certainly, the nucleus with its chromatin has long been considered the cell's most highly specialized center, at least physiologically and genetically, and it is this structure in which the iron is always found.

Dastre ('98-'99) says that a tissue like the liver, rich in iron, may contain as much as 1.5 parts iron in 10,000. It is possible that the nerve cells contain fully that much, if not more.

Regarding the relation of iron to the chromatin material, Macallum ('91) concluded that the iron-holding nuclein is the same as the chromatin—at least in the case of the maturing ovum. In lower vertebrates, he says, the iron which is present in them is received during the embryonic stage in combination with a nuclein, while, in the cat, there is a transference of chromatin from the maternal tissues to the foetal villi of the placenta, and the embryo receives, in this way, all the iron that it requires.

Later Macallum ('96) concluded that a substance to which iron is firmly held is a constant constituent of the nucleus of plant and animal cells, of the cytoplasm of non-nucleated organisms, and also of cells which possess rudimentary nuclei. The substance is not constant in molecular structure, but it occurs in the nuclein or nucleinic acid of cells in which it is probably held to an end carbon atom. Acid alcohols liberate this iron as a ferric salt which quickly becomes a molecular form. That it is in its ferric form is shown by the formation of the potassium ferro-ferri-cyanide or Prussian blue. Macallum claimed that chromatin is the antecedent of hemoglobin and has the capacity for absorbing and retaining oxygen. Therefore, alterations in the conditions of oxidation and reduction may be due to a reduction of the iron-containing nuclear constituent. In *Monotropa* none of the iron-reacting material found in the nuclei is derived from the cytoplasm, as may be shown, because there is often very little or no cytoplasm in the cells of coats of ovules of this plant, and yet the nuclei give an intense iron reaction. In muscle cells of larval *Amblystoma* in their transition stage, iron can be traced from the cytoplasm into the nuclei. Macallum suggests that it is possible that the iron-containing material in the cytoplasm of the gluten layer of germinating rye grain is the zymogen or prozymogen of the ferment. He observed diffusion of an iron-holding substance from the nucleus of the spermatozoid into its cytoplasm after it penetrated the ovule. This helps to support the view that new chromatin material in nerve cells is passed from the nucleus through its membrane into the surrounding cytoplasm, which was maintained by Scott. That the antecedent of ferments in the digestive secretions of Protozoa is an iron-holding chromatin is also maintained by Macallum, and he says that some yeast cells have eosin-staining bodies which show iron reaction. Probably these bodies are iron-containing oxyphilic substances.

In the work of Ascoli ('99) in which he found as much as 1 per cent of masked iron in a nucleinic acid, Macallum's view—that the organic iron is held to the nuclein or the nucleinic acid—is partly confirmed.

Certainly the iron is organically bound to the protein, because a 4 per cent sulphuric acid alcohol solution plus heat is necessary to unmask it.

Kappers ('21, p. 15) concluded that "The chromidial substance is a very complicated one. . . . This much, however, is certain, that it is an acid derivative (a compound of nucleinic acids with iron), and its acid character is also demonstrated by the fact that it can be colored only by a basic dye."

To the anatomist chromatin is a complex protein colloid containing nucleic acid to which some iron is bound and which stains readily with such basic dyes as thionin, methylene blue and toluidin blue. This chromatin is the chief constituent of the nucleus of all cells and such cytoplasmic materials as the Nissl bodies in nerve cells. Furthermore, it is believed to play an important part in cell division, growth, reproduction and metabolism.

To the physiologist the chromatin represents the storehouse for nutritive material because of the fact that it is depleted during activity of the cell and is replaced upon rest. But this view is not wholly justified because it certainly does not consider all the factors involved in cellular activity.

Just what the chemical and physiological processes are which take place in the neuron during metabolism and especially during the passage of the nerve impulse is a question difficult to answer. There are enough theories which will not be enlarged upon here. However, it seems well to give some of the explanations that have been offered in the past.

Schneider ('90) said that the nucleus seems to play the important rôle of taking up the required iron from the blood and then dispensing it as it is needed during the cell's metabolism.

Macallum ('92) said that iron in plant cells seems to be very necessary to life, since its depletion leads to the condition of chlorosis. This is questionable to some extent in animal life. Macallum went still further in his idea of the importance of iron when he said, "Suffice it to say that the fundamental life substance is an iron-containing compound and that, inferentially, the chemical processes underlying life, in other words life itself,

are to be referred to the constant oxidation and reduction of the iron of this compound."

Dastre ('98-'99) claimed that iron promotes oxidation of the organic matter; that it does so like an enzyme, without giving up itself; that iron readily combines with O_2 and Fe_2O_3 .

In 1904 Dastre still attached great importance to the iron in chromatin when he said that iron is one of the elements of living things, it enters readily into organic composites, and in plant and animal cells its chemical function seems to be that of acting as an agent of oxidation.

Porter ('95) also attempted to show that iron has a physiological rôle to play because, he says, it fixes O_2 and promotes oxidation in the tissues.

It was Mott ('21, p. 306) who said that if the iron of the nucleus acts as a catalyzer, as there is reason to believe, it may be hypothecated that when a stimulus arrives at the layer of intercalary neurons a catalasè is liberated from the nuclear material, acts upon the molecular oxygen attached to the oxidase granules, converts it into free atomic oxygen whereby physical and chemical changes occur, resulting in either a physiological junction of the processes of the intercalary neurons by amoeboid movement and multiple contacts with the processes of the first type of neurons or a process of combustion whereby the sugar is converted into energy thus serving as a source of stimulus to the next system of neurons.

That changes occur in the iron-containing materials of nerve cells has already been pointed out elsewhere in this paper and the figures support these views. These changes agree, for the most part, with the findings in regard to the Nissl substance in the cells that have been observed by other methods of study in which the iron-holding protein was not taken into account. Just what these changes in distribution of the iron-reacting substances mean as regards their relation to axone reaction it is difficult to say. That the iron is contained within or associated with the Nissl substance has been asserted by Mackenzie ('97), Macallum ('98) and Scott ('99), and in this study abundant proof of this is offered for the first time. Except for the smaller and

more peripheral Nissl granulations all the Nissl substance contains iron. Now, in the cell's reaction to axone injury (or, for that matter, injury to the cell body directly) both the Nissl granules and the iron-containing material become changed. The Nissl material becomes chromatolyzed and the iron disappears—so far as we know. In the light of the foregoing it is conceivable that, as the cell reacts to injury, the Nissl material might disappear entirely through hydrolysis of its complex protein molecules with which there may be associated some lipoids and carbohydrates, and, that this material is replaced by the cell's taking up more necessary material from the surrounding media as it survives the acute rise in metabolism.

But what happens to the iron-holding protein? It may be said that if the Nissl material is hydrolyzed the iron should be demonstrable in the cytoplasm just the same. This question can be met with the argument that the iron is not demonstrable as it comes into the cell either. Why, then, is it not possible that it passes out of the cell in a similar form? It may be suggested that in the increased metabolism following injury the iron-containing protein becomes divided into such fine particles, so widely dispersed and at a rate so slow that it is not visible by color reactions under apochromatic immersion lenses, and that, as the cell survives the acute reaction and tends toward the normal, this material again becomes aggregated. This view is supported by the fact that after chromatolysis of the Nissl material and dispersion of the iron-reacting material, there is an increase of the Nissl substance and iron-containing substance around the nucleus and an increase in iron-holding material within it. It may be that the iron-reacting masses are formed by the nucleus and become more abundant during the acute nuclear reaction.

Iron-containing protein disappears rapidly during the first four days, probably because at that time the cell is in its acute stage of reaction to injury. Perhaps the metabolic rate is increased and in the cell's reaction its available material is rapidly oxidized, and in so doing the iron-holding substance is oxidized also (fig. 25). But an attempt to explain this phenomenon can only be an hy-

pothetic one, since there is no more reason for such an explanation than there is for looking upon this material as a stored food substance like fat.

In the next three or four days still more of the organically bound iron is oxidized in the heightened metabolism of the cell and it becomes accumulated about the axone hillock (fig. 29). If iron passes into the hillock it must be in a form organically bound in such a way that it is not demonstrable by the methods at hand, or else it is ionized and diffused before fixation.

In the stage where the cell is hardly able to withstand the effects of the injury (about the twelfth day), practically all of the iron-containing substance becomes depleted in the cell apparently under the influence of active processes and in the direction of the axone hillock. At this stage it becomes a question whether the cell is going to live or if it must die (fig. 35). If the cell has the necessary vitality, its reaction toward the injury is arrested, it is going to live, and more iron material is gradually accumulated in the cell and finally the cell is regenerated to the point where it is again practically normal (fig. 70). If complete degeneration takes place, the cell dies.

Since the iron-reacting material is present in greatest amount in the side of the nucleus adjacent to the axone in the normal cell (fig. 5), in the injured cell just before the maximum degenerative effects have taken place (fig. 33) and again in the cells when the regenerative changes have begun to take place (fig. 70), we have another indication of the cell's polarity. Now what does this mean? It is possible that the dendrites and axone are of opposite electrical character and that the iron-containing material is thus collected on the axone side of the motor cell. Marfori ('92) showed that the iron obtained from ferratin from liver cells could be collected electrically at the negative pole. If the iron is positive it may be assumed that it is bound to a negative carbon atom in the protein molecule just as Macallum ('96) suggested. In further support of this view Macdonald, Macallum and Menton showed that there is also a content of potassium in and about the axone hillock, which is greater in amount than it is in any other part of the nerve cell, and potassium is electro-positive in character.

The cell shrinkage observed in the injured cells may be explained by the fact that during increased activity of the cell, hydrolysis takes place which results in an increase in CO_2 and H_2O in the cytoplasm. The CO_2 is carried away rapidly by the surrounding media and the blood, but the water remains, at least for a time. Then, when the cells are dehydrated with alcohols, the cytoplasm shrinks and shows the shrinkage areas in the fixed preparation. When the cells return to normal activity they do not show shrinkage after careful dehydration.

From the foregoing discussion it seems justifiable to look upon the iron-containing nucleoprotein as a distinct chemical compound in which changes in amount and distribution following the cell's reaction to mechanical injury are here studied for the first time. In a sense these changes may be regarded as normal activity of the living protoplasm. The changes under the above circumstances are, furthermore, essentially similar to those seen in nerve cells injured by excessive activity. The data presented in this paper afford the first proof that during the axone reaction there are definite chemical changes within the cell. The studies with dyes have been heretofore interpreted by some as indicating only physical changes in the cytoplasm. The present demonstration that most of the stainable substance of Nissl is an iron-containing substance makes it clear that the Nissl pictures of injured or fatigued cells indicate chemical as well as physical changes. When the struggle of the nerve cell to regenerate its axone has reached a climax at the end of the second week, a large amount of the iron-containing nucleoprotein has disappeared. There has likewise been an absolute decrease in organically bound iron. Excentricity of the nucleus and its usual changes in form described by investigators are the morphological evidences of the activities which will bring about the restoration. Ionized iron may be passing from the lymph through the cytoplasm into the nucleus, there to be incorporated into the protein molecule, or it may be bound, but the incoming stream being too slow or too small in amount to show, it is not demonstrable by this iron method. Or it may be that it is washed out or that it is too thin to see in fixed preparations. The treatment of the fresh cells

with potassium ferrocyanide and HCl might reveal it. However, this, as well as the effects of excess iron in the blood stream, should be studied both for the normal cells and such as are in various phases of axone reaction.

So fundamental a cell constituent as a nucleoprotein cannot be regarded as a mere reserve food material like stored glycogen. It is a chemically distinct substance, the reactions of which with other cytoplasmic elements are one phase of protoplasmic activity, perhaps one of the most important. The diffusion of nucleoproteins through the cytoplasm increases the contact areas enormously and should mean more rapid as well as more intense reactions.

The fact that some small Nissl granules do not show iron may be due to the particular iron-containing nucleoprotein having been hydrolyzed to the point where iron is no longer demonstrable, the same as in the condition where practically all the Nissl material and iron have disappeared.

CONCLUSIONS

1. The microchemical test for organically bound iron is reliable.
2. In the normal nerve cells of the hypoglossal nucleus of the rat microchemical tests show that there is organically bound iron in the nucleolus where it is most concentrated, in the oxychromatin of Heidenhain and in the chromophilic substance of the cytoplasm. Particular cells stained first by the Nissl method and then chemically tested for iron-containing substance present identical histological pictures except for the absence of iron material in some of the smaller Nissl granules and its presence in the acidophilic oxychromatin of the nucleus (figs. 84 and 85).
3. The behavior of the iron-holding protein was studied under two experimental conditions, first, after ligation of the hypoglossal nerve and, second, after tearing this nerve apart external to its foramen. That the continuity of the nerve fibers was interrupted by the ligation was shown by the degeneration of the distal moiety of the nerve, its electrical inexcitability and the atrophy of the musculature supplied.

4. During the first fifteen days after ligation of the nerve there is a gradual reduction in the amount of iron-containing material in the cytoplasm. In the first three days of this period this reduction is evident immediately around the nucleus, especially on the side nearest the axone, and the masses of iron-containing material increase in size in the peripheral parts of the cytoplasm. From the fourth to the eighth day these large masses fragment and scatter. Meantime there is a further reduction in the amount of cytoplasmic iron-reacting substance progressively from the nucleus toward the periphery. During this period iron-holding protein accumulates around the axone hillock which diminishes in size. During the period from the ninth to the fifteenth day practically the whole of the cytoplasm becomes free from iron-containing material except for occasional very small and widely scattered granules, and a further accumulation of iron-holding substance around the axone hillock, which meanwhile continues to diminish in size. From the sixteenth to the forty-fourth days the reparative changes take place. At first small granules of iron-reacting protein appear in irregular and scattered form close to the nuclear membrane from whence they become increased gradually toward the periphery of the cell. These granules of iron material gradually become more numerous, larger in size, and more regular in shape and distribution, and finally become most abundant in the zone of the cytoplasm midway between the nucleus and the cell wall, until the fifty-fourth day when their appearance in every respect is like that of the normal cell. It is seen, therefore, that in the reparative stages the lost iron is replaced, first in the vicinity of the nucleus and finally in normal distribution. No changes in the distribution of iron-reacting substance were observed in the dendrites. Neither was iron observed in the axones in any stage of the reaction to the injury.

5. After axone ligation the amount of iron-holding material in the nucleus diminishes during the first three days, after which it increases to an amount beyond the normal until the eighth day. This is followed by a diminution to below the normal amount to the fifteenth day at which time it is most marked. Between

the sixteenth and the forty-fourth days there are slight increments in some of the cells, but the return to normal amount and distribution of the nuclear iron-containing material is gradual up to the forty-fourth day. Throughout this period of axone reaction the iron-containing substance is nearly always more abundant in the side of the nucleus nearest the axone. No changes in the iron-reacting protein of the nucleolus were observed.

6. It is thus seen that the iron-containing material changes in distribution and decreases in amount during the degenerative stage which continues from the first up to the fifteenth day of axone reaction; that the maximum changes occur between the ninth and fifteenth days; that regenerative changes begin at the sixteenth day and continue up to the forty-fourth day of axone reaction; and that the cell recovers completely from the injury. So far as this investigation goes iron-reacting material passes from the nucleus into the cytoplasm where it accumulates about the axone hillock, but it is never seen to pass from the cytoplasm into the axone.

7. In those experiments where the axones were torn the reaction changes are slightly more accentuated and the stages are longer. The iron-containing material disappears from the dendrites, and in the cytoplasm it gradually diminishes from the first up to the twentieth days when it has completely disappeared. After the fifth day the reduction of cytoplasmic iron-holding substance is so great that the axone hillock can no longer be recognized.

8. In these preparations the nucleus shows a decrease in amount of iron-reacting material after the twentieth day which continues gradually up to the thirty-fifth day, when all of the iron-holding substance has disappeared, even from the nucleolus, and such cells are undoubtedly dead. In other words the nucleus undergoes degeneration later than the cytoplasm.

9. As in the case of the normal neurons (paragraph 1), identical cells stained by Nissl's method and subsequently tested for iron-reacting material show practically the same appearances in the cytoplasm throughout both series of axone reactions—those ligated and those torn (figs. 86 to 89). They also show that

the stainable substance of Nissl is, for the most part, an iron-containing protein.

10. The axone reaction involves not merely a change in the staining properties of the cytoplasm, but also an actual loss of iron organically bound to the protein molecule—thus a true chemical change.

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PLATE 1

EXPLANATION OF FIGURES

Figures 1, 2, 3 and 4 are photomicrographs of which figure 1 was enlarged $\times 50$ and figures 2, 3 and 4 were enlarged $\times 350$. Reduced one-third in reproduction. The tissue represented in figure 1 was fixed in Regaud's fluid and stained with Mallory's connective tissue stain. The tissues represented in figures 2, 3 and 4 were fixed in absolute alcohol plus 1 per cent strong ammonia and impregnated with AgNO_3 after the method of Ranson.

- 1 Cross section of tongue from rat after ligation of hypoglossal nerve showing decrease in size of tongue and atrophy of muscle fibers.
- 2 Central portion of ligated nerve showing normal axis cylinders.
- 3 Distal portion of ligated nerve showing degenerated axis cylinders.
- 4 Section through neuroma at point of ligation showing axis cylinders which have grown at random.



PLATE 2

Figures 5 to 23, inclusive, represent the amount, size of granules and distribution of the iron-containing material in the normal nerve cells of the uninjured side of the hypoglossal nucleus and were drawn with Zeiss apochromatic objective 1.5 mm., compensating ocular No. 12 and camera lucida at table level. Since they were reduced one-half they represent a magnification of 1,050 diameters. All of the preparations were fixed in 95 per cent alcohol and the microchemical coloration was obtained by the Macallum hematoxylin method.

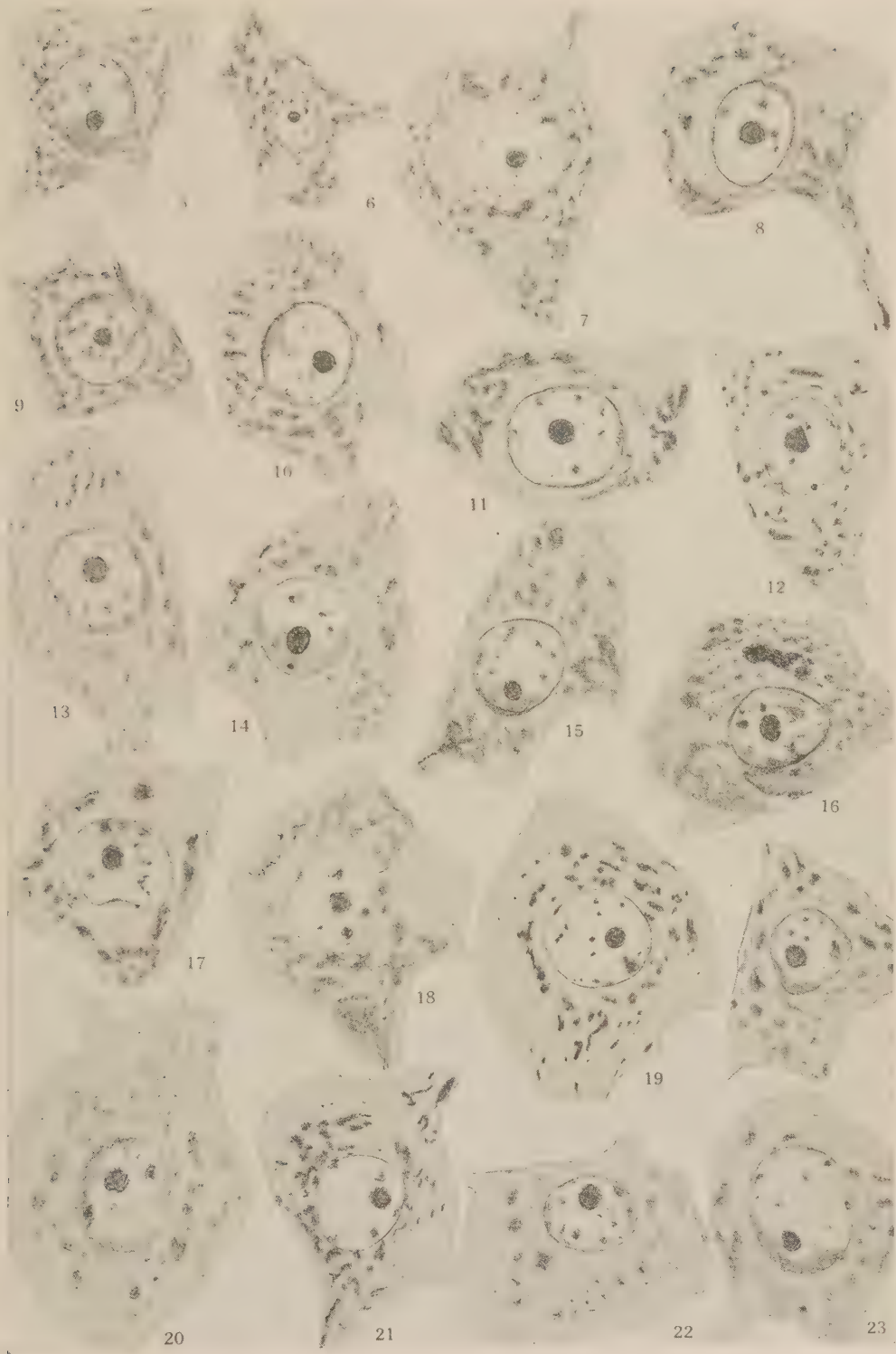


PLATE 3

EXPLANATION OF FIGURES

Figures 24 to 44, inclusive, represent changes in amount and distribution of iron in injured cells of the hypoglossal nucleus whose axones were ligated and were drawn with Zeiss apochromatic objective 1.5 mm., compensating ocular No. 12 and camera lucida at table level. They were reduced one-half and represent a magnification of 1,050 diameters. All the preparations were fixed in 95 per cent alcohol and colored microchemically with hematoxylin. In figures 26, 30, 32, 33 and 35 we have a typical central chromatolysis pictured.

- 24 Hypoglossal nucleus nerve cell after injury duration of 1 day.
- 25 After injury duration of 2 days.
- 26 After injury duration of 3 days.
- 27 After injury duration of 4 days.
- 28 After injury duration of 5 days.
- 29 After injury duration of 6 days.
- 30 After injury duration of 7 days.
- 31 After injury duration of 8 days.
- 32 After injury duration of 9 days.
- 33 After injury duration of 10 days.
- 34 After injury duration of 11 days.
- 35 After injury duration of 12 days.
- 36 After injury duration of 13 days.
- 37 After injury duration of 14 days.
- 38 After injury duration of 15 days.
- 39 After injury duration of 16 days.
- 40 After injury duration of 17 days.
- 41 After injury duration of 18 days.
- 42 After injury duration of 19 days.
- 44 After injury duration of 21 days.

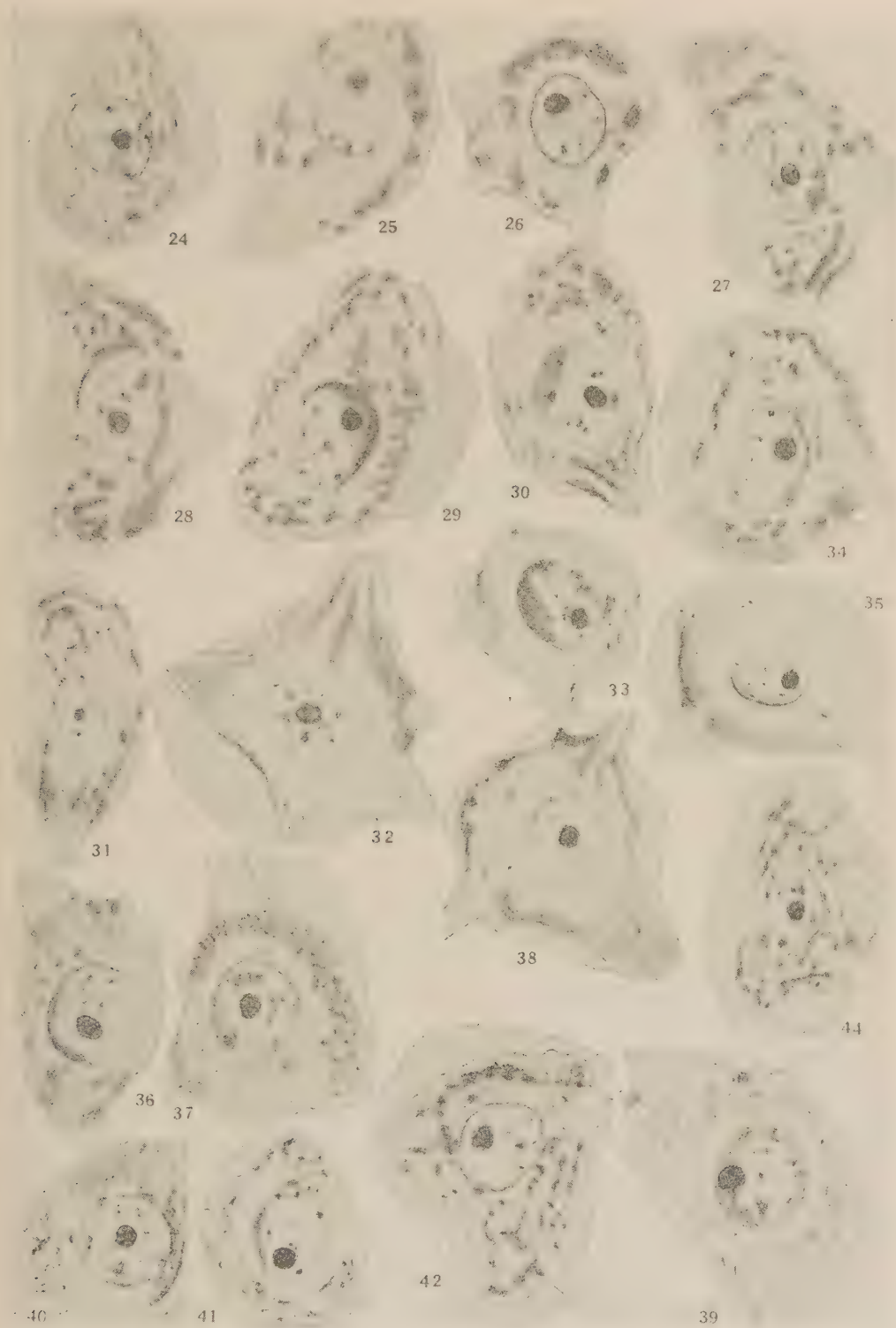


PLATE 4

EXPLANATION OF FIGURES

The pathologic changes, method of drawing, magnification, fixation and coloring of illustrations represented by figures 43 to 63 are like those described in plate 3.

- 43 After injury duration of 20 days.
- 45 After injury duration of 22 days.
- 46 After injury duration of 23 days.
- 47 After injury duration of 24 days.
- 48 After injury duration of 25 days.
- 49 After injury duration of 26 days.
- 50 After injury duration of 27 days.
- 51 After injury duration of 28 days.
- 52 After injury duration of 29 days.
- 53 After injury duration of 31 days.
- 54 After injury duration of 32 days.
- 55 After injury duration of 33 days.
- 56 After injury duration of 34 days.
- 57 After injury duration of 36 days.
- 58 After injury duration of 37 days.
- 59 After injury duration of 38 days.
- 60 After injury duration of 39 days.
- 61 After injury duration of 40 days.
- 62 After injury duration of 41 days.
- 63 After injury duration of 42 days.

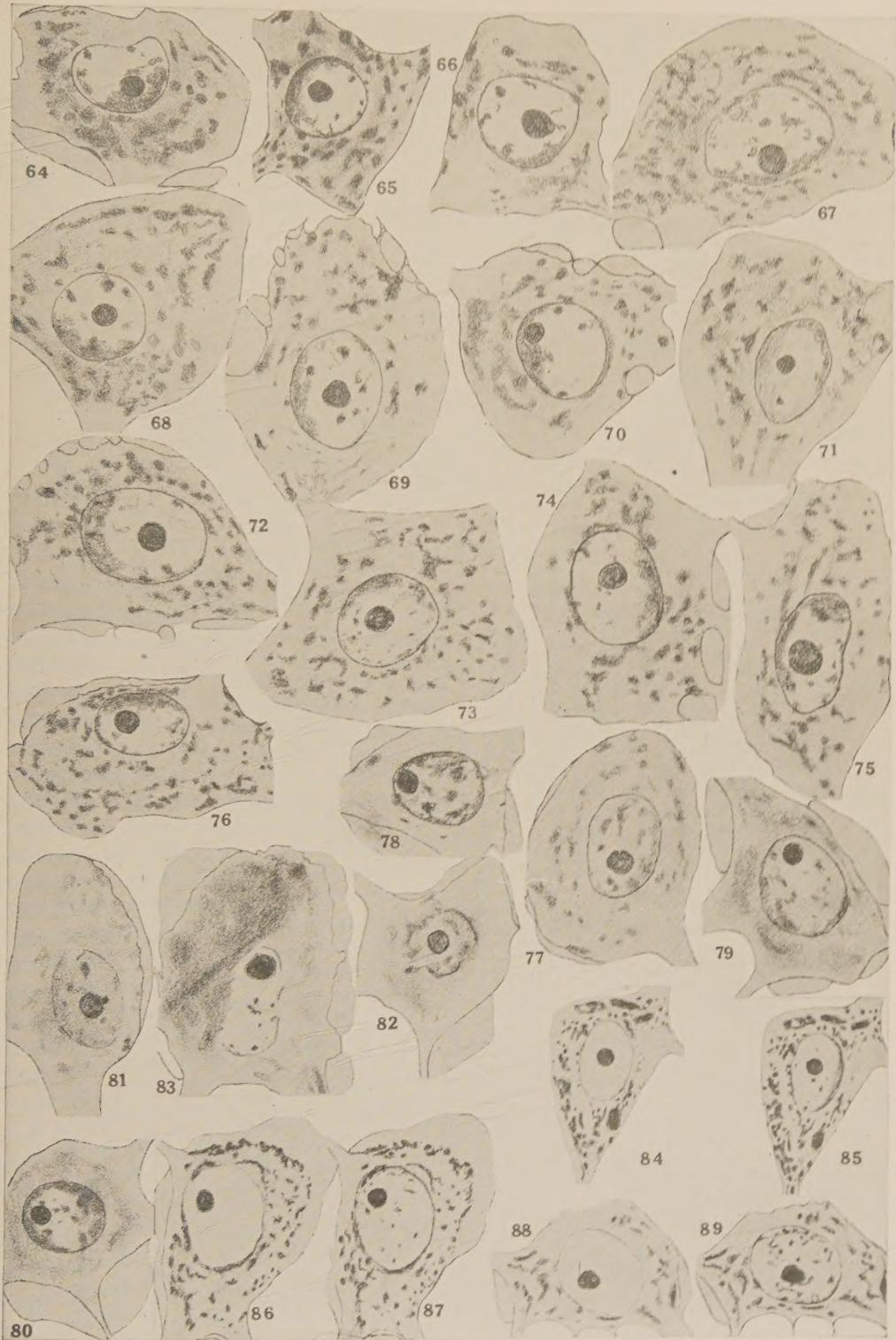


PLATE 5

EXPLANATION OF FIGURES

Figures 64 to 89, inclusive, were treated like those in plates 3 and 4 except that figures 84, 86 and 88 were stained by the Nissl method to show the Nissl substance before they were subjected to the unmasking of the iron and the demonstration of this material by the hematoxylin method as represented in figures 85, 87 and 89.

- 64 After injury duration of 43 days.
- 65 After injury duration of 44 days.
- 66 After injury duration of 45 days.
- 67 After injury duration of 46 days.
- 68 After injury duration of 47 days.
- 69 After injury duration of 48 days.
- 70 After injury duration of 49 days.
- 71 After injury duration of 50 days.
- 72 After injury duration of 2 months.
- 73 After injury duration of 3 months.
- 74 After injury duration of 4 months.
- 75 After injury duration of 5 months.
- 76 After injury duration of 6 months.
- 77 Five days after tearing axones.
- 78 Ten days after tearing axones.
- 79 Fifteen days after tearing axones.
- 80 Twenty days after tearing axones.
- 81 Twenty-five days after tearing axones.
- 82 Thirty days after tearing axones.
- 83 Thirty-five days after tearing axones.
- 84 Normal nerve cell stained by Nissl method.
- 85 Same cell destained with alcohol after which the iron was unmasked and then treated with hematoxylin for the iron reaction.
- 86 Cell whose axone was ligated. Stained by Nissl method.
- 87 Same cell showing iron reaction after having been destained and treated with acid-alcohol for unmasking purposes.
- 88 Nerve cell whose axone was torn. Stained by Nissl method.
- 89 Same cell destained and in which the iron was unmasked and then colored by the hematoxylin method.



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